Screening and Isolation of Lactic Acid Bacteria to Produce Lactic Acid at High Concentration

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List of abbreviations

DCW	Dry cell weight
g	Gram
g/L	Gram per Litre
hr	Hour
min	Minutes
OD	Optical density
rpm	Revolution per min
°C	Degree Celsius
ml	Millilitre
l	Litre

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ABSTRACT

This study was carried out with the aim to screen and isolate lactic acid bacteria (LAB) from natural sources to produce lactic acid (LA) at high concentration. The screening and isolation of LAB from natural sources is important to obtain useful and genetically stable strains for industrial application. A LAB was isolated from local fermented cabbage from Kuching, Sarawak. An important parameter as the optimum temperature to achieve 100 g/l of LA in batch fermentation mode was investigated. The fermentations were performed in 3 l jar fermentor with a working volume of 2 l using glucose and yeast extract as carbon and nitrogen source respectively. The results showed that the optimum temperature to produce the highest concentration of dry cell weight and LA was 37°C. Using an inoculum of biomass at 1.57 g/l the LA concentration achieved was 101.78 g/l complaining with aim the study. The $_{max}$ was 0.69hr^{-1}, the volumetric productivity was 4.2 g/lhr and the specific productivity was 1.7 g/ghr at 37°C. This study also showed that the temperature played an important factor to overproduce LA and temperatures higher than 37°C were not favourable for lactic acid fermentation. We can conclude that this isolated has potential to be used for industrial application.

Key Words: temperature, batch fermentation system, lactic acid, fermented cabbage, glucose.

ABSTRAK

Kajian ini dijalankan dengan tujuan untuk menyangankan dan mengasingkan laktik asid bakteria (LAB) dari sumber asal untuk menghasilkan laktik asid (LA) pada kepekatan tinggi. Penyangan dan pengasingan organisma dari sumber asal adalah penting untuk mendapatkan strain yang berguna dan stabil dari segi genetik untuk kegunaan industri. LAB diasingkan dari jeruk kobis tempatan dari Kuching, Sarawak. Parameter penting iaitu suhu optimum untuk mencapai 100 g/l LA melalui sistem fermentasi secara batch telah dikaji. Fermentasi tersebut dijalankan dalam 3 l balang fermentasi dengan 2 l jumlah bekerja menggunakan glukosa dan ekstrak yis sebagai sumber karbon dan nitrogen secara berturut-turut. Keputusan fermentasi menunjukkan suhu optimum untuk menghasilkan kepekatan tertinggi berat cell kering dan LA ialah 37°C. Menggunakan inokulum biomass pada 1.57 g/l, kepekatan LA yang dicapai ialah 101.78 g/l yang menunjukkan objektif kajian ini telah dicapai. μ_{max} ialah 0.69 hr^{-1}, penghasilan volumetrik ialah 4.2 g/lhr dan penghasilan spesifik ialah 1.7 g/ghr pada 37°C. Kajian ini juga menunjukkan suhu memainkan peranan penting dalam penghasilan LA dan suhu tinggi daripada 37°C tidak digemari untuk fermentasi LA. Kita boleh membuat kesimpulan bahawa strain ini mempunyai potensi untuk kegunaan industri.

Kata kunci: suhu, sistem fermentasi "batch", laktik asid, jeruk kobis, glukosa

1.0 INTRODUCTION

The screening and isolation of microorganism from natural sources is important to obtain useful and genetically stable strains for industrial application. LABs are divided into 16 genera, some 12 of which are active in food context. They are Gram-positive, catalase negative organisms and either rod, cocci (spherical) or coccobacilli in shape. Mostly are mesophilic, but some can grow at refrigerator temperatures (4°C) and as high as 45°C. Generally they prefer a pH in range 4.0 to 4.5, but certain strains can tolerate and grow at pH above 9.0 or as low as 3.2. They need preformed purines, pyrimidines, amino acids and B vitamins. LAB is used substrate-level phophorylation to gain their energy because they do not posses tricarboxylic acid cycle or haem-linked electron transport system. LA and other organic acids are produced by LAB act as natural preservative and flavour enhancer. Hence, reduce spoilage and enhance taste of the food products. Besides, probiotics LAB such as *Lactobacillus* and *Bifidobacterium* species have expanded their application into health foods (Salminen & von Wright, 1993; Heller, 2001).

Their metabolism or fermentation process can be classified as either homofermentative or heterofermentative. In homofermentative, LA represents 95 % of the total end products, while, in heterofermentative, acetic acid, ethanol and carbon dioxide are produced alongside LA. However, LAB that is valuable for industrial potential should be homofermentative, such as they produce only LA. Heterofermentative LAB is considered lower yielding strains because carbon dioxide and other types of organic acids are produced together with LA. Besides, recovery of LA and subsequent purification is easier in homofermentative process. Other desirable properties for an industrially useful LAB are the synthesis of pure isomers and tolerance to high product concentration and high

temperature (42°C or higher) as these external conditions themselves would be useful to minimize contamination of the culture by other microorganisms (Tsai *et al.*, 1993).

The problem statement of this study is low concentration of LA production by heterofermentative LAB. Besides, it needs high nutrition requirements which may increase the production cost. Finally, LA production inhibits the LAB growth. For industrial application the concentration of LA that make a process economically feasible is around 100 g/l. Lower concentration has the problem to remove more water in downstream process to recover the LA. Hence, the objective of this study is to screen and isolate microorganism to produce LA at 100 g/l in 24 hrs. The hypothesis is natural LAB can produce LA at concentration higher than 100 g/l in short time as 24 hrs.

Fermented cabbage was used as source for the isolation of LAB. The isolates were screened for catalase activity and Gram-staining. Followed by, study of their performances for LA production profiles. The effect of temperature on the growth of the isolates was also focus of this study.

A study by Kimoto *et al.* (2004) found that a total of 411 LAB of genus *Lactococccus* were isolated from plant materials. Out of the strains, 27 strains were identified as *Lactcocccus lactis*. The plant materials were fermented vegetables, silage and plants.

2.0 LITERATURE REVIEW

2.1 Lactic acid

LA (2-hydroxypropanoic acid) is an organic hydroxy acid which occur abundantly in nature. There are two optical forms of LA, D- and L- LA. LA can be produced commercially either by chemical synthesis or by microbial fermentation. In chemical synthesis from petroleum feedstock, only racemic DL form of LA is produced. On the other hand, optically pure isomers of LA can be produced through fermentation process.

2.2 Applications of lactic acid

LA has been widely used in a various industries. The usages of LA in various industries are as below:

2.2.1 Food industry

LA is naturally present in many foodstuffs. It is formed by natural fermentation in products such as cheese, soy sauce, sourdough, meat products and pickled vegetables. Besides, LA also widely used in bakery products, beverages, meat products, confectionery, dairy products, salads dressings, ready meals and so on. LA in food usually acts as either pH regulator or preservative. It is also used as a flavouring agent.

2.2.2 Pharmaceutical industry

The primary functions of LA in pharmaceutical are as pH-regulator, metal sequestration, chiral intermediate and as a natural body constituent in pharmaceutical products.

2.2.3 Leather and textile industry

Besides, LA is also used in leather and textile industry where acidity is required and where its properties offer specific benefits. The examples are the manufacture of leather and textile products, computer disks and car coating.

2.2.4 Animal feed

LA is a commonly used as an additive in animal nutrition. It has health promoting properties which can enhance the performance of farm animals. LA can be used as an additive in food and/or drinking water.

2.2.5 Biodegradable plastics

LA is the principal building block for Poly LA (PLA). PLA is a biobased and biodegradable polymer that can be used for producing renewable and compostable plastics. This kind of plastic is a good option for substituting conventional plastic produced from petroleum oil because of low emission of carbon dioxide apart reducing the increasing amount of solid waste nowadays.

2.3 Effects of temperature on the growth of lactic acid bacteria

Temperature is one of the most important factor that affecting bacterial growth rate. Each 10°C increase in temperature will approximately doubles chemical reaction rate. However, this relationship only holds for a defined temperature range. As an instance, growth rate fails to increase cell death at low temperature. Hence, the fermenter should have an adequate provision for temperature control. Heat is generated by microbial activity and

agitation. Heat removal or addition may not be required if the heats generate a temperature that is optimum for the fermentation processes. Unfortunately, usually either additional heating or removal of the excess heat would be required. Temperature control may be considered at laboratory scale, and pilot and production scale.

Microorganisms are classified based on optimum growth temperature. This is due to the types of enzyme in the cell for biochemical reactions. The three categories of microorganisms are psychrophiles, mesophiles and thermophiles. The first types are psychrophiles. These grow best at cold temperatures (optimal growth 10° to 20°C) and are found in arctics. The second types are mesophiles. These optimally grow at moderate temperatures (optimal growth at 20 to 40°C). Most bacteria that have adapted to humans are mesophiles and grow best near human body temperature (37°C). The last types are thermopiles. Thermophiles grow best at high temperatures (optimal growth at 50 to 60°C). These are found in hot springs.

2.4 Lactic acid bacteria in fermented cabbage process

Fermented cabbage can take 3 weeks and ideally at temperatures below 20°C. The stage of microbial involvement in vegetable fermentation consists of four stages: the start stage, the primary fermentation, the secondary fermentation, and the post-fermentation. In the start stage, a range of Gram-positive and Gram-negative bacteria is present. The second stage is the primary fermentation, the point at which LAB inhibits growth of most bacteria in acid conditions. Hence, LAB and yeast are able to thrive. During the secondary fermentation stage, LAB growth is now inhibits by low pH, but no yeasts are growing fermentatively. In the last post-fermentation stage, oxidative bacteria, moulds and yeasts are growing on

the surface of open tanks. However, no growth is observed in seal anaerobic tanks if pH is low enough and salt concentration high enough.

LAB constitutes a relatively small proportion of the total bacterial count in the fermented cabbage and comprises five major species: *Enterococcus faecalis, Leuconostoc mesenteroides, Lactobacillus brevis, Pediococcus cerevisae* and *Lactobacillus plantarum*. These microorganisms represent the most significant contributor to the fermentation although with low level. A low salt concentration (2 %) and low temperature (18°C) favour heterofermentative microorganisms. Conversely, a high salt concentration (3.5 %) and high temperature (32°C) promote homofermentative fermentation. The normal sequence is heterofermentation followed by homofermentation. Glucose and fructose are main sugars in cabbage and small amount sucrose. In anaerobic fermentation, they are converted to acetic acid, mannitol, ethanol and carbon dioxide in the first week. After a week or so, the fermentation is continued by homofermenters because the brine becomes too acidic for heterofermenters. Production of LA continues until all the sugars are consumed and the pH has dropped from around 6 to 3.4.

2.5 Batch fermentation

Figure 1 shows the microbial growth phase in batch culture system. The batch growth can be divided into four phases which are lag, exponential or log, stationary and death.

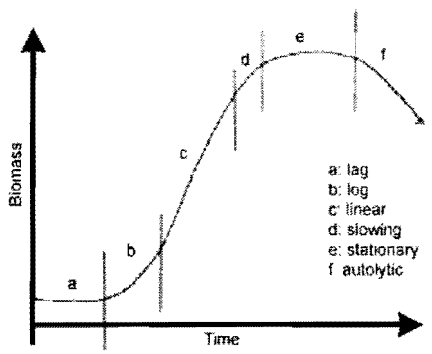

Figure 1: Microbial growth phase in batch culture system (MicrobiologyBytes, 2009).

Batch culture system represents growth in a closed system or environment which contains limited amount of nutrient medium. Scragg (1991) points out that it is operated under the optimum conditions of temperature, pH and redox potential. As shown in figure 1, after inoculation, the culture enters lag phase. During lag phase, the sizes of the cells are increased but not their number. After that, the cultures enter log or exponential growth phase in which the cells divide at a maximal rate and their generation time reaches maximum. Then, in stationary phase, the growth is stationary due to depletion of the nutrients and the accumulation of inhibitory end products in the medium. Eventually, the stationary phase of bacterial population culminates into death phase in which the viable bacterial cells begin to die.

Batch reactor is easy to set up and run. Besides, the initial conditions can be standardized and the operating conditions can be easily control without risk of contamination to the culture if proper sampling procedures are applied.

3.0 MATERIALS AND METHODS

3.1 Sample collection

Two samples of fermented vegetables were obtained from retail markets in Kuching, Sarawak. The samples were held under refrigeration at 4°C until examination that takes place within 48 hrs.

3.2 Isolation and screening of lactic acid bacteria

Approximately 10 g of each sample was weighted. The sample was vigorously shaken with 90 ml of sterile distilled water in sterile bottle. A sample of 1 ml was inoculated into 100 ml broth of 10 % glucose and 5 % yeast extract in a 250 ml Erlenmeyer flask. The flask was incubated at 37°C for 24 hrs in static condition. After 24 hrs of incubation, 1 ml of the culture was transferred into fresh medium. The flask was incubated at 37°C for 12 hrs in static condition. Then, aliquot of the culture from the flask was diluted serially for 10 times. Approximately 0.1 ml of broth and 0.9 ml of sterile distilled water was well mixed in sterile centrifuge tube. From this, 0.1 ml was spread evenly on 10 % glucose and 5 % yeast extract agar plates. The plates were incubated at 37°C for 48 hrs. Next, glistening colonies were picked from the glucose and yeast agar plate by sterile loop. The isolates were subcultured in 20 ml broth in universal bottle with two duplicate. One of the universal bottles from each isolate was incubated at 37°C for 24 hrs. The next day, the pH was checked and the isolated strain with the lowest pH was further purified for further study. The isolate was maintained in 20 % glycerol for storage at -20°C. Consequently, the isolate was tested for catalase by placing a drop of 3 % hydrogen peroxide solution on the

cells. Immediate formation of bubbles indicates the presence of catalase in the cells. Later, only catalase-negative isolate was Gram-stained.

3.3 Growth and lactic acid production profiles

Strain with the lowest pH was selected to generate their growth and LA production profiles. Repeated batch fermentation was performed in 2000 ml fermentor containing basal glucose and yeast extract medium with pH 7.4. Approximately 100 ml of 18 hrs culture was used as inoculums. The inoculum was prepared as the basal medium but with only 10 g/l glucose and 5 g/l yeast extract. Then, the flask was incubated at 37°C at static conditions. The cells were spun down and washed twice with 0.85 % saline before being transferred into the 2000 ml fermentor. The test temperatures were 37°C, 38°C and 40°C with pH 7.4. Then, fermentation at 37°C was further tested as it give the optimum growth and LA production profiles with basal medium of 100 g/l glucose and 5 g/l yeast extract with pH 7.4. At the end of the fermentation, the culture was diluted with sterile tap water to certain optical density (OD) and 10 ml was removed for dry cell weight (DCW).

3.4 Analytical techniques

3.4.1 Assay of lactic acid

The LA production was assayed based on sodium hydroxide (NaOH) consumption. Approximately 10M NaOH was pumped into the fermentor when LA was produced which indirectly dictates LA production. The amount of LA was determined as illustrated in the following equation:

$$LA(g/l) = \frac{(W_i - W_f)x0.69}{V}$$

Where: W = Initial weight of NaOH (g)

W= Final weight of NaOH (g)

V= Final volume at the end of the fermentation (l)

The factor 0.69 came from the next equation:

$$Factor = (\text{concentration of NaOH}) x \left(\frac{\text{Mass of NaOH pumped}}{\text{Density NaOH)}} \right) x \left(\frac{\text{Lactic acid Molweight}}{\text{NaOH Molweight}} \right)$$

3.4.2 Determination of biomass concentration by DCW

The broth sample was diluted to certain OD with sterile tap water. Approximately 10 ml of the broth sample was put in 100 ml centrifuge tube. The broth was centrifuged at 10, 000 rpm for 10 mins. Then, the supernatant was discarded and 10 ml of 0.2 M HCl was added in the tube. The cells were resuspended and centrifuged again at 10,000 rpm for 10 mins. Next, the supernatant was discarded and 10 ml of HCl was added again. After that, the tube was centrifuged again at 10,000 rpm for 10 mins. Later, the supernatant was discarded and distilled water was added in the tube to resuspend the cells. Then, the resuspend cells were filter with the filter membrane (0.45 μm in diameter, cellulose) and oven dry for 3 days at 60°C until the weight was constant. DCW was determined as follow:

$$DCW(g/l) = \frac{(A+B)-A}{M}$$

Where: A= Weight of dried filter (g)

B= Weight of Cells (g)

M= Volume of sample (l)

4.0 RESULTS AND DISCUSSION

4.1 Isolation and screening of lactic acid bacteria for lactic acid production

The samples from fermented cabbages produced a total of 8 isolates with potential to produce LA. However, only two isolates possessed capability to decrease the pH of the broth in a short period of time. The isolate with the fastest capability to produce LA was chosen for further study over various temperatures.

Figure 2 shows the isolate was cultured in a broth and spread plate on glucose and yeast extract agar plate.

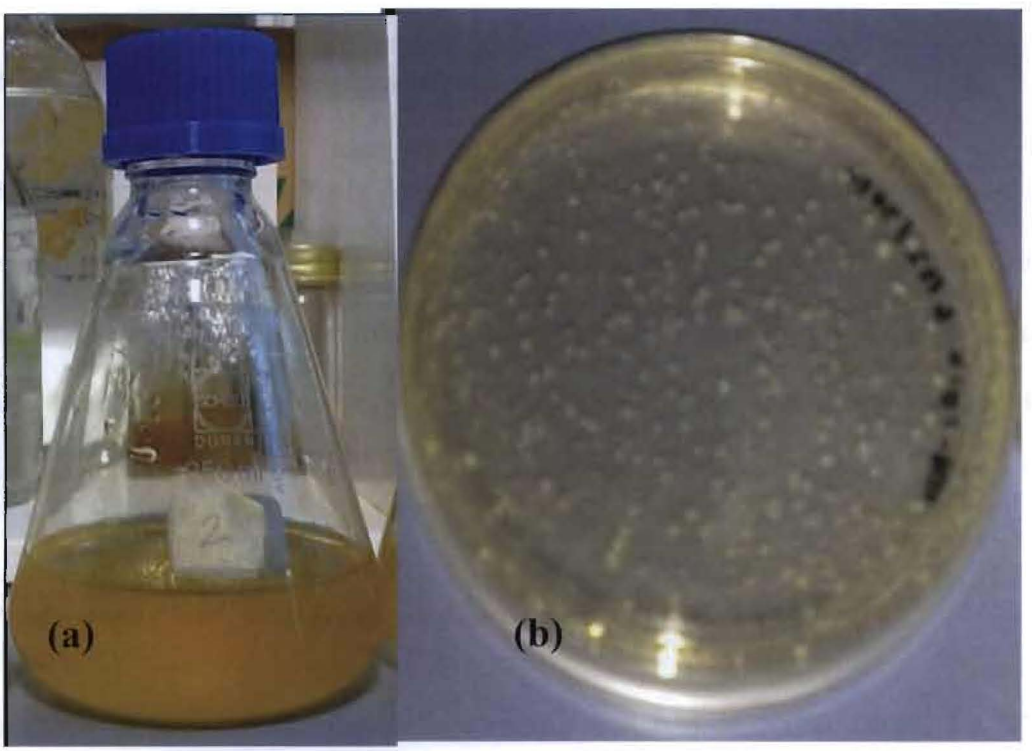

Figure 2: Isolate cultured on (a) broth (b) glucose and yeast extract agar plate.

4.2 Description of isolate characteristics on agar medium

Table 1 shows the isolate was morphologically observed on agar medium and documented by using various parameters.

Table 1: Description of isolate morphology on agar medium.

Colony morphology	
Shape	Circular
Size (diameter in mm)	1
Elevation	Convex
Surface	Smooth and glistening
Edge	Entire
Pigment	No pigment
Opacity	Opaque
Consistency	Sticky
Odour	Absent

4.3 Biochemical characteristics identification of lactic acid bacteria isolate

Catalase test and Gram stain were performed on the isolate. The result of the catalase test shows that the isolate was catalase negative because no bubble was produced. Meanwhile, the result of the Gram stain shows that the isolate was Gram positive microorganism because blue stain microorganism was shown. Therefore, the isolate was confirmed as LAB.

4.4 Production of lactic acid by repeated batch fermentation

Butler (2004) pointed out that most cells in culture grow best at 37°C and at pH 7.4. When the cells were subjected to a temperature slightly lower than the 37°C optimum, then the growth rate was reduced but the cells were not damaged. However, higher temperatures of 39°C to 40°C will destroy the cells. Thus, it is very important to be sure that the

temperature does not increase in the incubator. The figure 3 shows the average and standard deviation for the biomass production in these fermentations.

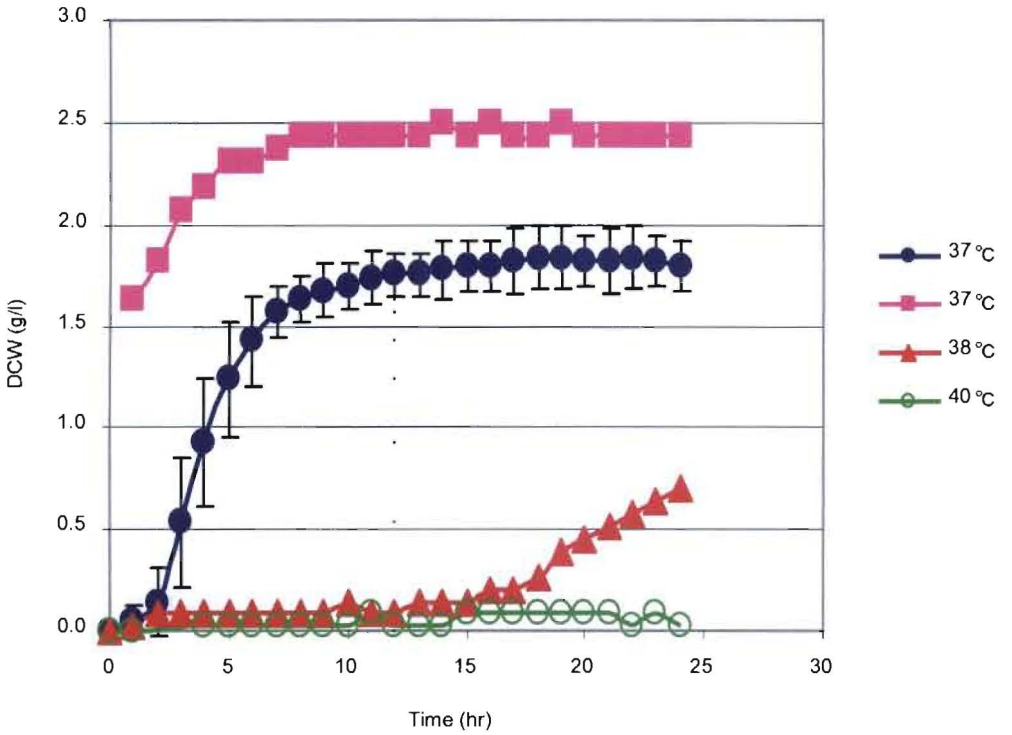

Figure 3: Biomass produced by the lactic acid bacteria isolate.

Repeated batch fermentation was done on the LAB isolate over various temperatures. The initial biomass of the fermentations was 0.10 g/l with pH 7.4 under various temperatures. It was found at 37°C was the optimum temperature for growing and LA production for the isolated strain. Therefore the fermentation at 37°C was done for four times. Three replicates at 37°C of fermentations were done with an initial biomass concentration of 0.10 g/l. As shown in figure 3, it was obvious that by increasing the biomass concentration was possible to increase the productivity of the system. Therefore, the last fermentation was done with biomass 1.57 g/l as initial inoculum of biomass in order to achieve the objective of this study. Fermentation at 37°C relatively shows faster reproduction of cells with the

highest biomass value among the test temperatures. At 6 hrs of fermentation, the biomass was with an average of 0.45 g/l. While, at 12 hrs of fermentation, the average of the biomass was 1.76 g/l. During 18 hrs of fermentation, the biomass was with an average of 1.82 g/l. At 24 hrs of fermentation, the biomass was with an average of 1.83 g/l.

Moreover, fermentations at 38°C were carried out to study the effect of the temperature on the cell growth. Initially one of the fermentation was used to increase the biomass at this temperature (data not showed). The fermentations were performed with an initial cell concentration of 0.10 g/l. Nevertheless, fermentation at 38°C shows longer lag phase with lower biomass value. At 6 hrs of fermentation, the biomass was with an average of 0.19 g/l. While, at 12 hrs of fermentation, the average of the biomass was 0.33 g/l. During 18 hrs of fermentation, the biomass was with an average of 0.58 g/l. At 24 hrs of fermentation, the biomass was with an average of 0.76 g/l.

However, fermentation at 40°C shows the LAB isolate was unable to grow. At 6 hrs, 12 hrs, 18 hrs and 24 hrs of fermentation shows the biomass was 0.16 g/l.

The faster reproduction of cells fermented at 37°C was proven by the shorter lag phase as shown in figure 3. As previously mention, during lag phase, the cells increase in size and weight rather than increase in numbers. Hence, it was a non-productive period of a fermentation process. It was often desirable to minimize or control the duration of the lag phase.

According to Lee (2006), lag phase can be divided into two types. The first type is apparent lag. Apparent lag phase is occurred when a portion of culture grow at maximum

rate while the rest fail to grow. While, true lag occurs when the culture not able to grow at its maximum rate initially due one of the following factors which are change in nutrient, change in culture conditions, presence of an inhibitor, spore germination and inoculums effect.

The first factor is changed in nutrient. The same medium formulation was used throughout this study. The medium formulation was glucose as the carbon source and yeast extract as the nitrogen source. It is always desirable to adapt an inoculum in the same medium as in the final culture system to minimize the duration of the lag phase. Besides, cells from a nutrient starved inoculums could experience substrate-accelerated death when they are inoculated into a nutritionally rich medium. As an example, the rapid flux of the growth-limiting substrate often the carbon led to unbalanced enzymatic activities and disruption of cellular metabolic control. The rapid decrease in the intracellular level of some essential metabolites will cause loss in viability. Hence, during inoculum preparation in this study, the maximum time for the fermentation was 24 hrs to avoid nutrient starvation.

The second factor is changed in culture conditions. Lee (2006) mentions, in the studies growth parameters in batch culture system, cultures are often started with inoculums of the same source and then incubated at different conditions such as various incubation temperatures. Cells incubated at conditions that deviate from the original culture conditions of the inoculum may experience a lag phase. Some cultures may even fail to grow in the conditions that deviate too much from the original culture condition. Thus, the outcome of the study is cell history dependent and could lead to bias conclusions. It is therefore desirable to preculture the cells in some particular culture conditions before they are used

as inoculum to study the effect on cell growth. Hence, in this study, the culture condition of the inoculum was set to be 37°C.

The third factor is presence of an inhibitor. Lee (2006) states that presence of an inhibitor may affect the culture. Hence, a period of time may be required to reduce the amount of an inhibitory substrate or an inhibitory medium component to a concentration which permits maximum growth rate. In some cases, the product of inoculums may inhibit growth. In addition, Scragg (1991) mentions that slower increase in biomass maybe due to accumulation of toxic material in broth. In addition, Jolhery (2001) reported that this effect maybe due to accumulation of LA. As an example, members of *Lactobacillaceae* when transferred from an anaerobic to aerobic medium may accumulate hydrogen peroxide (H_2O_2) in the cells. This could happen when a heavy inoculums of *lactobacilli* with low oxygen partial pressure in the original culture is incubated into a freshly prepared medium with oxygen partial pressure at air saturation. Growth would only be possible after the synthesis of the enzyme peroxidise and removal of H_2O_2. This means by increasing the inoculum size would prolong the lag period as the production of H_2O_2 is proportional to the cell concentration. Hence, during this study, the inoculum was spun down and washed twice with 0.85 % saline before being transferred into the fermentor. This may reduce the presence of inhibitor in the initial inoculation.

The fourth factor is spore germination. If the inoculum is made up of spores, vegetative growth is only possible after germination of spores. However, LAB do not produce spore. Therefore, the fourth factor cannot be applied in this study.

The last factor is the inoculum effect. Lee (2006) says that the length of growth lag is also affected by the physiological stage such as the age and size of the inoculum. The inoculum at the initial stage of the exponential growth phase and at the stationary phase may prolong the lag phase. Nevertheless, the inoculum at the late exponential growth phase or by adding intermediates of citric acid cycle may eliminate lag phase. Growth lag may represent "priming" of various essential metabolic pathways before substrates are metabolized at maximum rates for conversion into biomass. Bujang (2000) claimed that 50 % of organic carbon will be converted to biomass under aerobic conditions, whereby only 5 % will be converted into biomass under anaerobic conditions.

In addition, Butler (2004) says that length of lag phase is depended on the culture medium formulation, initial concentration and state of the cells. Low inoculation density and viability of the inoculums tend to create longer lag phase. This may occur if subculture is delayed. High density of cells with good viability may eliminate lag phase altogether.